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### Authors

Yang, Zhaoqing  
Zhang, Zaixin  
Sun, Xiaodong  
et al.

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## Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Yunnan Province, China

Zhaoqing Yang<sup>1, 5</sup>, Zaixin Zhang<sup>2</sup>, Xiaodong Sun<sup>2</sup>, Wenlin Wan<sup>1</sup>, Long Cui<sup>5</sup>, Xiang Zhang<sup>3</sup>, Daibin Zhong<sup>4</sup>, Guiyun Yan<sup>4</sup> and Liwang Cui<sup>5</sup>

1 Parasitology Department, Kunming Medical College, Kunming, Yunnan, China

2 Yunnan Institute of Parasitic Diseases, Simao, Yunnan, China

3 Mengla county hospital, Mengla, Yunnan, China

4 Program in Public Health, College of Health Sciences, University of California, Irvine, CA, USA

5 Department of Entomology, The Pennsylvania State University, University Park, PA, USA

### Summary

Resistance of *Plasmodium falciparum* to chloroquine (CQ) is determined by the mutation at K76T of the *P. falciparum* chloroquine resistance transporter (*pfcr*) gene and modified by other mutations in this gene and in the *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene. To determine the extent of polymorphisms in these genes in field *P. falciparum* isolates from Yunnan province of China, we genotyped the *pfcr* codon 76, *pfmdr1* codons 86 and 1246. Our results showed that although CQ has been withdrawn from treating falciparum malaria for over two decades, 90.3% of the parasites still carried the *pfcr* K76T mutation. In contrast, mutations at *pfmdr1* codons 86 and 1246 were rare. Sequencing analysis of the *pfcr* gene in 34 parasite field isolates revealed CVIET at positions 72–76 as the major type, consistent with the theory of Southeast Asian origin of CQ resistance in the parasite. In addition, two novel *pfcr* haplotypes (75D/144Y/220A and 75E/144Y/220A) were identified. Real-time polymerase chain reaction was used to determine *pfmdr1* gene amplification, which is associated with mefloquine resistance. Our result indicated that in agreement with that mefloquine has not been used in this area, most (>90%) of the parasites had one *pfmdr1* copy. Genotyping at two hypervariable loci showed relatively low levels of genetic diversity of the parasite population. Meanwhile, 28.4% of cases were found to contain mixed clones, which favour genetic recombination. Furthermore, despite a unique history of antimalarial drugs in Yunnan, its geographical connections with three malarious countries facilitate gene flow among parasite populations and evolution of novel drug-resistant genotypes. Therefore, continuous surveillance of drug resistance in this area is necessary for timely adjustment of local drug policies and more effective malaria control.

**keywords** malaria parasite, chloroquine resistance, *Plasmodium falciparum* chloroquine resistance transporter gene, *Plasmodium falciparum* multidrug resistance 1, genetic diversity

### Introduction

The emergence and spread of malaria parasites resistant to many antimalarials, particularly chloroquine (CQ) and pyrimethamine (PY)–sulphadoxine (SD), is largely responsible for the recent global resurgence of malaria, which severely hampers our capacity to roll back malaria (Snow *et al.* 2004; Olliaro 2005). In China, malaria is still a major public health problem with most of the cases concentrated in two subtropical provinces, Yunnan and Hainan. Yunnan is located in Southwest China, bordering three malaria-endemic countries, Myanmar, Laos and Vietnam. Malaria here is perennial with peak

transmission occurring from June through October, coincident with the rainy season. Malaria cases in Yunnan are concentrated in several counties near the borders and about one-third are due to *Plasmodium falciparum* infections. Here, CQ resistance in *P. falciparum* appeared in the early 1970s and spread rapidly. By the early 1980s, it was replaced by PY–SD to treat falciparum malaria (Liu *et al.* 2005).

Since then, longitudinal surveys have been carried out in these two malarious provinces and a general decline of CQ resistance was noticed (Liu *et al.* 2005). In Hainan, *in vitro* microtests demonstrated a drop in the rate of CQ-resistant *P. falciparum* from 97.9% in 1981 to 60.9% in 1991 and

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26.7% in 1997 (Liu *et al.* 1995,2005). Correspondingly, 4-week *in vivo* observations detected a decrease of CQ-resistant parasites from 84.2% in 1981 to 40% in 1991 and 18.4% in 1997. Although a similar trend was observed in Yunnan, the decrease of CQ resistance was insignificant and over 90% of *P. falciparum* remained CQ resistant in 1992 (Yang *et al.* 1994). A more recent survey in 2002 indicated that CQ resistance rate in Yunnan was still above 70% (Liu *et al.* 2005). While this trend of declining CQ resistance as its cessation in many endemic areas suggests a possible rotation of CQ in the future (Kublin *et al.* 2003), such a decision requires close regional monitoring because evolution of CQ resistance after the removal of drug pressure seems to differ considerably among parasite populations.

Chloroquine resistance is manifested by impaired CQ uptake by the parasite vacuole, which is correlated with mutations in several genes. A single mutation K76T in the *P. falciparum* chloroquine resistance transporter gene (*pfcr*) has been demonstrated to be a major determinant of CQ resistance in *P. falciparum* (Fidock *et al.* 2000; Sidhu *et al.* 2002; Lakshmanan *et al.* 2005). All *P. falciparum* clinical samples that are resistant to CQ contain the K76T mutation (Djimde *et al.* 2001). As at least five founder mutations of K76T have been detected worldwide (Wellems & Plowe 2001; Wootton *et al.* 2002; Chen *et al.* 2005), genetic backgrounds of the resistant strains differ greatly. Other epistatic markers such as *P. falciparum* multidrug-resistant 1 (*pfmdr1*) gene also affect the final outcome of CQ resistance (Reed *et al.* 2000; Babiker *et al.* 2001). Five single nucleotide polymorphisms (SNPs) of *pfmdr1* N86Y, Y183F, S1034C, N1042D and D1246Y were identified in field isolates from different regions of the world. Some correlation studies showed that 86Y was associated with CQ resistance in isolates from the Old World, whereas the C-terminal mutations were found in isolates from South America (Hayton & Su 2004). A recent study demonstrated that N1042D of *pfmdr1*, a prevalent mutation in South America, contributes to quinine instead of CQ resistance, suggesting that *pfmdr1* mutations can affect parasite susceptibility to a wide range of antimalarials depending on the parasite's genetic background (Sidhu *et al.* 2005). While the effect of *pfmdr1* point mutations might affect the parasite's sensitivity to different drugs, recent correlation studies demonstrated that *pfmdr1* amplification is responsible for resistance to mefloquine (Wilson *et al.* 1993; Price *et al.* 1999,2004; Nelson *et al.* 2005).

Currently, the K76T mutation is widely used as a reliable marker for CQ-resistance in epidemiological studies (Wongsrichanalai *et al.* 2002). In different continents, this marker has been shown to correlate with

*in vivo* CQ resistance (e.g. Jelinek *et al.* 2002; Berens *et al.* 2003; Daily *et al.* 2003). A recent molecular analysis of *P. falciparum* samples from Hainan province collected from 1978 to 2001 showed a continuous decrease in the prevalence of K76T, consistent with the increased CQ sensitivity determined by *in vitro* and *in vivo* drug assays (Wang *et al.* 2005). In Yunnan, most malaria drug resistance surveillance relied on an *in vitro* microtest described by Rieckmann *et al.* (1978) and *in vivo* monitoring (Yang *et al.* 1994,1995,1997), whereas molecular epidemiological studies of drug resistance have not been carried out. Here, we report the population diversity of *P. falciparum* parasites in Yunnan and evaluated the prevalence of polymorphisms at *pfcr* and *pfmdr1*. Our results showed a relatively low level of genetic diversity of the parasites with 28.4% mixed-strain infections. Despite the withdrawal of CQ from treating falciparum malaria for over two decades, the *pfcr* K76T mutation still had over 90% prevalence. In contrast to the high prevalence of 86Y mutation in certain Southeast Asian *P. falciparum* samples, *pfmdr1* mutations at codons 86 and 1246 were very rare in the Yunnan samples. Consistent with that mefloquine has never been used here; the level of *pfmdr1* gene amplification was low.

## Materials and methods

### Samples

*Plasmodium falciparum* field isolates were collected in 2003–2004 in two counties of Yunnan Province. Twenty samples were collected from Gengma county, Lincang district bordering Myanmar and 69 samples were from Mengla county, Xishuangbana district, bordering Laos and Myanmar (Figure 1). Both areas had a similar level of malaria endemicity, within the range of 14–44 malaria incidence per 10 000 people in 2000. The human subject protocol for this study as part of the ongoing malaria epidemiological surveys of the Yunnan Institute of Parasitology was approved by the provincial review board. Parasite samples were collected from patients with uncomplicated *P. falciparum* infections before drug treatment. Initial diagnosis was carried out by microscopic examination of Giemsa-stained thick blood films. For each sample, approximately 200 µl of finger-prick blood were spotted on a piece of Whatman 3M filter paper and air-dried. The dried filters were stored in individual plastic bags at –20 °C until DNA extraction. Parasite DNA was extracted from the blood filters using either the Chelex boiling method (Plowe *et al.* 1995) or the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA).



**Figure 1** Map of Yunnan Province showing the locations (stars) where parasite samples were collected.

#### Parasite genotyping at merozoite surface protein 1 (*msp1*) and glutamate-rich protein (*glurp*) loci

Nested polymerase chain reaction (PCR) was performed to differentiate the three molecular types of *msp1* in the polymorphic block 2 as described (Kaneko *et al.* 1997). First PCR was performed with conserved outer primers followed by three individual nested PCR using primer pairs specific for allelic variant families of the *msp1* block 2. The central repeat fragment of *glurp* was amplified similarly by nested PCR using primers described earlier (Roper *et al.* 1998). Sizes of the PCR products were estimated following electrophoresis on agarose gels. Alleles with similar sizes (e.g. 350 and 375 bp) were binned around an average size. The multiplicity of infection (MOI), or number of genotypes per infection, was calculated as the highest number of genotypes at any of the two genes.

#### Determination of prevalence of *Plasmodium falciparum* chloroquine resistance transporter K76T, *Plasmodium falciparum* multidrug-resistant 1 N86Y and D1246Y mutations

Determination of polymorphisms at these codons was performed by using a similar PCR–restriction fragment length polymorphism (PCR–RFLP) method. Primers and conditions for *pfcr*t codon 76, *pfmdr*1 codons 86 and

1246 were previously described (Djimde *et al.* 2001). *Apo* I, which cuts the K76 codon, was used to distinguish wild-type from the mutant. To determine the polymorphisms at the *pfmdr*1 codons 86 and 1246, *Afl* III and *Eco* RV were used, which cut the 86Y and 1246Y mutants, respectively (Duraisingh *et al.* 1997; Syafruddin *et al.* 2005).

#### Determination of other mutations in the *Plasmodium falciparum* chloroquine resistance transporter genes

To identify other *pfcr*t mutations in the Yunnan isolates, we selected 34 samples of the 76T genotype for sequencing of the *pfcr*t fragments. These samples were pre-determined by genotyping *msp1* and *glurp* to be of single-clone infections in order to avoid complications during direct sequencing of the PCR products. In brief, three fragments of the *pfcr*t gene were amplified from each sample using primers described by Chen *et al.* (2003). PCR products were purified by filtration columns (Qiagen) and sequenced directly using the BigDye terminator chemistry on an automated ABI sequencer. The accuracy of sequences was confirmed by sequencing from both ends of the PCR products.

#### Determination of *Plasmodium falciparum* multidrug-resistant 1 copy number by real-time polymerase chain reaction analysis

An increased *pfmdr*1 copy number has been associated with *in vitro* and *in vivo* mefloquine resistance (Wilson *et al.* 1993; Price *et al.* 1999, 2004; Nelson *et al.* 2005). To assess the *pfmdr*1 copy number in the Yunnan *P. falciparum* isolates, we performed real-time PCR analysis for 60 single-clone infections on the basis of the genotyping results. Real-time PCR was performed as described (Ferreira *et al.* 2006) using FastStart SYBR Green mix (Roche) with primers for *pfmdr*1 and a reference gene on chromosome 14 encoding fructose-bisphosphate aldolase (*fba*) (*fba*-F: GGGAATATTCTCGAAGTAA and *fba*-R: CGGTGATGGTGTITTTAG). Data were analysed using the OpticonMonitor Analysis Software and the threshold cycle,  $C_t$ , was defined as the cycle number at which the change of fluorescence in the reaction exceeds 10 SDs above the background. Background fluorescence was calculated as the mean fluorescence between cycle 3 and 7 (Miao *et al.* 2006). For each sample, the  $C_t$  value was determined for both *pfmdr*1 and *fba* genes. All reactions were performed in triplicate and the result was rejected if the SD value was above 0.5. Reactions performed with serial dilutions of DNA from the 3D7 clone, known to harbour a single copy of *pfmdr*1 and *fba*, were used to

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calibrate the  $C_t$  values from the field samples. Copy number of *pfmdr1* gene was calculated using the  $2^{-\Delta\Delta C_t}$  method:  $\Delta\Delta C_t = (C_{t-mdr1} - C_{t-fba})_X - (C_{t-mdr1} - C_{t-fba})_{3D7}$ , where X is the test sample (Ferreira *et al.* 2006) (Table 3).

## Results

### Parasite population diversity

Parasites were genotyped at the *msp1* and *glurp* loci to evaluate the population diversity at the two study sites in Yunnan. Both genes were successfully amplified from 88/89 samples (Table 1). While the result showed the presence of all three *msp1* variant types (K1, MAD20 and RO33), the predominant allele family was MAD20, which was originally defined in an isolate from Papua New Guinea (PNG) (Tanabe *et al.* 1987). This suggests that MAD20 may be the prevalent type circulating in certain malarious regions of Asia. While only one size of approximately 150 bp was detected for the MAD20 type, the K1 type was more variable with four sizes (200, 300, 350 and 375 bp) being detected. The RO33 type was the least abundant at both sites. Genotyping *msp1* also detected samples containing different allele families indicative of mixed-strain infections, which accounted for 21.1% and 30.4% of the samples from Gengma and Mengla, respectively. Comparing with a total of six alleles found for *msp1*, parasites at the *glurp* locus were less diverse with only four alleles observed and their distributions were also biased. The frequencies for the 375, 475, 550 and 800 bp alleles were 18.3%, 52.1%, 21.1% and 8.55%, respectively. The MOI of Mengla samples (1.3) was slightly higher than that of Gengma samples (1.21) (Table 1).

### *Plasmodium falciparum* chloroquine resistance transporter allele types and their prevalence in Yunnan province

*In vitro* microtests indicated that although CQ was abandoned for one and two decades, the prevalence of CQ

resistance in *P. falciparum*-endemic regions of Yunnan province remained high (Yang *et al.* 1994; Liu *et al.* 2005). Consistent with this notion, the prevalence of *pfprt* K76T mutation was 78.3% and 93.3% in *P. falciparum* samples from Gengma and Mengla counties, respectively. Overall, the K76T mutation had a 90.3% prevalence in the Yunnan samples.

In addition to the major CQ-resistance determinant K76T, there are at least 10 other *pfprt* mutations associated with CQ-resistant isolates from various geographical regions. To assess the levels of polymorphisms in 10 of these codons, we employed a strategy used by Chen *et al.* (2003) to amplify short fragments of *pfprt* from 34 single clones for sequencing. Consistent with the result from PCR-RFLP analysis, sequencing confirmed the presence of K76T mutation in all 34 samples. A total of five haplotypes were observed with the haplotype *a* as the most prevalent, accounting for 70.6% of the sequenced samples (Table 2). This haplotype was also the major one observed in CQ-resistant parasites from Asia and Africa (Wootton *et al.* 2002). Compared with the wild-type CQ-sensitive sequence of the 3D7 clone, all the *pfprt* sequences from Yunnan had 5–6 mutant codons. Sequences at positions 72–76 were either CVIET or CVIDT. None of the predominant sequences found in the Philippines (CVMHT) or Papua New Guinea (SVMNT) was observed in Yunnan. Whereas the A220S mutation is associated with the K76T mutation in most CQ-resistant parasites, 9/34 sequenced Yunnan samples with K76T mutation had the wild-type 220A (Table 2). A similar observation was made in a subset of samples from the Philippines (Chen *et al.* 2003). Furthermore, two abundant mutations A144T and L160Y identified in samples from the Philippines (Chen *et al.* 2003) and the A144F mutation observed in other Southeast Asian samples (Durrand *et al.* 2004) were not found in the Yunnan samples. Instead, a novel A144Y mutation was found in 9/34 sequences. Interestingly, all the A144Y mutants were linked with the wild-type A220. Collectively, this study identified two novel *pfprt* haplotypes 75D/144Y/220A and 75E/144Y/220A.

**Table 1** Parasite diversity at the *msp1* site

| Sites  | K1 (%) | MAD20 (%) | RO33 (%) | Number of samples† | Number of isolates | MOI  | Number of mixed infections (%) |
|--------|--------|-----------|----------|--------------------|--------------------|------|--------------------------------|
| Gengma | 21.7   | 69.6      | 8.7      | 19/20              | 23                 | 1.21 | 4 (21.1)                       |
| Mengla | 31.1   | 67.8      | 1.1      | 69/69              | 90                 | 1.30 | 21 (30.4)                      |
| Total  | 29.2   | 68.1      | 2.7      | 88/89              | 113                | 1.28 | 25 (28.4)                      |

MOI, multiplicity of infection.

†Number of samples successfully tested/total number of samples.

**Table 2** Sequence polymorphisms in *pfert*

| Origin                 | Isolate | Amino acid in <i>pfert</i> at position |    |    |    |    |     |     |     |     |     |
|------------------------|---------|--|----|----|----|----|-----|-----|-----|-----|-----|
|                        |         | 72                                     | 74 | 75 | 76 | 97 | 144 | 160 | 220 | 271 | 371 |
| Yunnan,<br>China       | 3D7     | C                                      | M  | N  | K  | H  | A   | L   | A   | Q   | R   |
|                        | a (24)  | C                                      | I  | E  | T  | H  | A   | L   | S   | E   | I   |
|                        | b (7)   | C                                      | I  | D  | T  | H  | Y   | L   | A   | E   | R   |
|                        | c (1)   | C                                      | I  | D  | T  | H  | Y   | L   | A   | E   | I   |
|                        | d (1)   | C                                      | I  | E  | T  | H  | Y   | L   | A   | E   | R   |
| Thailand               | e (1)   | C                                      | I  | E  | T  | H  | A   | L   | S   | E   | R   |
|                        | a       | C                                      | I  | E  | T  | H  | A   | L   | S   | E   | I   |
| Cambodia               | b       | C                                      | I  | E  | T  | L  | A   | L   | S   | E   | I   |
|                        | a       | C                                      | I  | E  | T  |    |     |     | S   |     |     |
| PNG                    | b       | C                                      | I  | D  | T  |    |     |     | S   |     |     |
|                        | c       | C                                      | M  | N  | T  |    |     |     | A   |     |     |
|                        | d       | C                                      | T  | N  | T  |    |     |     | S   |     |     |
|                        | a       | S                                      | M  | N  | T  | H  | A   | L   | S   | Q   | R   |
| Solomon<br>Philippines | b       | S                                      | M  | N  | T  | H  | A   | L   | A   | Q   | R   |
|                        | a       | C                                      | M  | N  | T  | H  | T   | Y   | A   | Q   | R   |
|                        | b       | S                                      | M  | N  | T  | H  | T   | Y   | A   | Q   | R   |
| Brazil                 | c       | C                                      | I  | E  | T  | H  | A   | L   | S   | E   | I   |
|                        | a       | S                                      | M  | N  | T  | H  | A   | L   | S   | Q   | R   |
| Ecuador                |         | C                                      | M  | N  | T  | H  |     |     | S   | Q   | R   |
| Colombia               |         | C                                      | M  | E  | T  | Q  |     |     | S   | Q   | T   |

Number in parenthesis indicates the number of isolates with that respective haplotype.

### *Plasmodium falciparum* multidrug-resistant 1 single nucleotide polymorphisms and copy number

In sharp contrast to the prevalence of K76T mutation in the Yunnan *P. falciparum* samples, only one isolate (<1%) had the *pfmdr1* N86Y mutation, while none was mutated at codon 1246 (Table 3). To rule out that the result obtained by PCR–RFLP analysis was due to incomplete enzyme digestion, we randomly selected 10 PCR products from each *pfmdr1* fragment for sequencing, which further confirmed the presence of wild-type sequences at the two codons (data not shown).

From 60 single-clone infections, the *pfmdr1* copy number was successfully determined in 53 samples, 44 of which were from Mengla county. When rounded to the closest integer, 90.6% isolates had a single copy *pfmdr1*, 7.5%

had two copies, and only one sample (1.9%) had three copies (Table 3). Interestingly, all these five isolates with amplified *pfmdr1* were from Mengla.

### Discussion

The widespread resistance to CQ and other antimalarials occurring in Southeast Asia has fostered the deployment of artemisinin-based combination therapy for falciparum malaria. Beginning in 2005, artemisinin-based combination therapy (dihydroartemisinin–piperaquine, Artekina) began to be the choice of *P. falciparum* therapy in Yunnan. Since widespread resistance to piperaquine has been observed (Yang *et al.* 1995), suitability of this partner drug for artesunate in this area is questioned. Meanwhile, declining resistance to CQ observed in *P. falciparum* in many malarious regions after its withdrawal suggests a possibility of future reintroduction of the drug. This consideration requires better understanding of the evolution of resistance in the absence of the drug pressure.

While drug resistance is expected to drop after the removal of selective pressure, this process can vary greatly between locations. More surprisingly, the two closely located provinces in China also experienced quite different paces in the evolution of CQ resistance although CQ was withdrawn almost concomitantly from the two provinces

**Table 3** Copy number and frequencies of mutations in *pfmdr1*

| Polymorphisms |       |
|---------------|-------|
| N86Y          | 0.9%  |
| D1246Y        | 0     |
| Copy number   |       |
| 1X            | 90.6% |
| 2X            | 7.5%  |
| 3X            | 1.9%  |

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two decades ago. Whereas a significant decline of CQ resistance was observed in Hainan following the cessation of CQ use (Liu *et al.* 1995; Wang *et al.* 2005), CQ resistance in Yunnan province remained high (Yang *et al.* 1997). Our molecular analysis of the major genetic determinant of CQ, the K76T mutation in *pfcr*, also supports the results from the *in vitro* microtests. Although the exact mechanism remains to be elucidated, we speculate that several factors may have contributed to the persistence of the K76T mutation in the Yunnan *P. falciparum* populations.

First, vivax malaria accounts for approximately 2/3 of all malaria cases in Yunnan and CQ is still the recommended drug for treating vivax malaria. In such *P. vivax*-*P. falciparum* sympatric areas, mixed-species infections are common and can reach as high as 20% (Snounou *et al.* 1993). Moreover, some of these co-infections are often diagnosed as vivax malaria by conventional microscopic examination and treated with CQ, when cryptic CQ-resistant *P. falciparum* infections emerge after treatment (Mayxay *et al.* 2001). Undoubtedly, CQ selective pressure on *P. falciparum* is retained in areas of falciparum-vivax co-existence. This factor may be responsible for the slow decline of *P. falciparum* CQ resistance in countries and regions of the Mekong Delta (Berens *et al.* 2003; Lim *et al.* 2003; Durrand *et al.* 2004; Congpuong *et al.* 2005).

Second, unlike Hainan Island that is isolated from other malarious regions, Yunnan borders three Southeast Asian countries, where malaria is highly endemic and CQ resistance levels are high (Labbe *et al.* 2001; Ngo *et al.* 2003; Dittrich *et al.* 2005; Hatabu *et al.* 2005). Genetic exchange and spread of resistant genotypes likely play an important role in maintaining CQ resistance in this area.

Third, deployment of structurally similar drugs with cross-resistance will slow down the decline of resistance. A prominent example is cross-resistance between CQ and amodiaquine (Holmgren *et al.* 2006). Although amodiaquine has never been used for malaria control in Yunnan, *P. falciparum* is highly resistant to this drug (Yang *et al.* 1997). In malaria-endemic areas of China, another quinoline drug, piperazine has been used extensively in the light of CQ resistance, and widespread resistance to this drug has been observed (Yang *et al.* 1995). While cross-resistance between the two drugs has not been clearly established (Basco & Ringwald 2003), the fact that they both contain similar structural components suggests that extensive deployment of piperazine may have imposed a positive selection pressure on *pfcr* mutations. Last, while development of drug resistance generally has fitness cost (Walliker *et al.* 2005), the evidence for reduced fitness in CQR parasites is circumstantial, which may vary from population to population. It is possible that the acquisition

of CQR alleles in the studied parasite populations in Yunnan might not result in reduced fitness as observed elsewhere (Kublin *et al.* 2003; Wang *et al.* 2005); thus the mutations would persist even in the absence of drug pressure. Future detailed studies of the genetic backgrounds of parasites in this area are needed for the elucidation of resistance to quinoline drugs.

Molecular and population studies suggest that CQ resistance in *P. falciparum* has evolved independently in at least four regions: Southeast Asia, PNG, and two in South America (Wellems & Plowe 2001; Wootton *et al.* 2002). The recent detection of other novel *pfcr* alleles in the Philippines suggests the possibility of additional founder events (Chen *et al.* 2003). A cluster of mutations in *pfcr* amino acids 72–76 distinguishes three major haplotypes in the global *P. falciparum* populations: the wild-type CVMNK, CVIET found in Asia/Africa, SVMNT associated with PNG and South America (Wootton *et al.* 2002). Besides these major haplotypes, others have been associated with different geographical populations of the parasite. For example, SVIET, CVIKT, CVIDT and CVTNT have been found in Southeast Asia (Nagesha *et al.* 2003; Lim *et al.* 2003). It is interesting that the SVMNT type, originally considered to be associated only with the PNG/South America populations (Mehlotra *et al.* 2001), was recently found to be the predominant haplotype in the Philippines (Chen *et al.* 2003), India (Vathsala *et al.* 2004) and Iran (Ursing *et al.* 2006). Our analysis of 34 samples from Yunnan identified two haplotypes CVIET (76%) and CVIDT (24%). That the former is the major haplotype present in Asia is consistent with the theory of Southeast Asian origin of the CQ-resistant parasites. The observation of the east-to-west increase of CQ resistance in Cambodia provides stronger support of this theory (Lim *et al.* 2003). As acquisition of CQ resistance is likely a stepwise process, the N75D and N75E mutations observed in the present and earlier studies might be different steps [AAT(N) → GAT(D) → GAA(E)] in the evolution of *pfcr* (Lim *et al.* 2003; Mitra *et al.* 2006). It is noteworthy that the N75E mutants also exhibit much higher IC<sub>50</sub> values to CQ than the N75D mutants (Durrand *et al.* 2004). While the significance of other *pfcr* mutations is less well understood, the A220S mutation has been tightly linked with the K76T mutation in most of the global CQ-resistant lines. Our analysis of the Yunnan *P. falciparum* isolates revealed that 25/34 of the isolates sequenced had such an association, whereas approximately 25% of the K76T isolates were wild-type at A220. The 76T/220A combination was also found as the main type in a subset of samples from the Philippines (Chen *et al.* 2003). The simultaneous finding of A144T and L160Y mutations in these samples led the authors to hypothesize that these might be compensatory

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mutations that confer CQ resistance in the absence of the A220S mutation. Interestingly, we did not observe the A144T and L160Y mutations in the Yunnan samples. Instead, our analysis of limited samples found that the 76T/220A haplotype was always associated with the A144Y mutation. The role of these new *pfcr* haplotypes in CQ resistance awaits further investigations.

The history of antimalarial drug usage in Yunnan is distinctive from those of neighbouring countries and other parts of the world. As a result of extensive CQ resistance in *P. falciparum* in late 1970s, this drug was withdrawn from treating falciparum malaria in 1983 in Yunnan. After replacement with PY-SP for a short while, artemisinin drugs were introduced and gradually became the mainstay of malaria therapy. Other drugs that have been used widely in bordering countries such as mefloquine have not been used in this region. Such a unique history of antimalarial drug usage probably has shaped the current parasite populations with different genetic backgrounds.

Of particular interest is the *pfmdr1* gene encoding an ABC transporter, which is a possible modulator of drug resistance in *P. falciparum*. Its effect in modulating resistance to CQ and other antimalarials probably depend on the genetic background of the parasite (Reed *et al.* 2000; Sidhu *et al.* 2005). Many field studies aiming to establish a statistical association of the widespread N86Y mutation in Asia and Africa with CQ resistance have yielded conflicting results (Wellems & Plowe 2001). Recent studies with Southeast Asian strains of *P. falciparum* also failed to demonstrate the N86Y mutation in CQ resistance, but established the role of *pfmdr1* amplification in mefloquine resistance (Price *et al.* 1999, 2004; Pickard *et al.* 2003; Nelson *et al.* 2005). In contrast to the high prevalence of 86Y mutation in some parts of Southeast Asia such as Indonesia (Syafuruddin *et al.* 2005), we found that mutations at codons 86 and 1246 were very rare in the Yunnan *P. falciparum* population. This result is congruent with similar findings of other *P. falciparum* populations from the Mekong Delta (Congpuong *et al.* 2005; Khim *et al.* 2005; Wang *et al.* 2005). It is likely that *pfmdr1* mutations may not play an important role in CQ resistance in this large region. Furthermore, real-time PCR analysis showed that less than 10% of the parasites from Yunnan had the *pfmdr1* gene amplification, which contrasted with 54% of isolates at the Thai-Myanmar border (Price *et al.* 2004). Again, this difference can be explained by the distinct mefloquine use histories in the two countries. Whereas mefloquine has never been used in Yunnan and parasites here are generally susceptible to this drug, it has been widely deployed in Thailand since 1985 (Yang *et al.* 1997; Sattabongkot *et al.* 2004). The lack of large geographical barriers between the two sites may facilitate

the spread of resistant parasites, which may account for the low-level *pfmdr1* amplification observed in the Yunnan parasite population.

Genetic diversity of the malaria parasite at a given region is correlated with the local transmission intensity. Drug selection, however, tends to diminish genetic diversity, especially near the resistance-determinant locus (Wootton *et al.* 2002). In agreement with Yunnan as a malaria hypoendemic region, genetic diversity at the hypervariable sites *msp1* (six alleles) and *glurp* (four alleles) was lower than what was observed previously in a similarly hypoendemic region in western Thailand (nine *msp1* and seven *glurp* alleles) (Paul *et al.* 1998) and in hyperendemic regions such as Tanzania (17 *msp1* alleles) (Babiker *et al.* 1994). The low number of *msp1* and *glurp* alleles and the predominance of a CQ haplotype may indicate that parasite population in this region is genetically homogeneous, which could be the result of the CQ-selective sweep. Nevertheless, despite low transmission intensity, the observed near 30% mixed-strain infections in Yunnan favour genetic recombination. In addition, its geographical connections with several malarious countries and the resultant frequent population migration across borders ensure large regional gene flows. Therefore, continuous surveillance of drug resistance is needed to extend the life span of individual antimalarial drugs and help local authorities develop more appropriate malaria control policies.

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**Corresponding Author** Liwang Cui, Department of Entomology, The Pennsylvania State University, 501 ASI Building, University Park, PA 16802, USA. Tel.: +1 814 863 7663; Fax: +1 814 865 3048; E-mail: luc2@psu.edu

#### Analyse moléculaire de la résistance à la chloroquine chez *Plasmodium falciparum* dans la province de Yunnan en Chine

La résistance du *Plasmodium falciparum* à la chloroquine (QC) est déterminée par la mutation K76T sur le gène transporteur de résistance à la chloroquine (*pfcr*) et modifiée par d'autres mutations sur ce gène et sur le gène de la multi résistance de *P. falciparum* (*pfmdr1*). Afin de déterminer sur le terrain l'ampleur du polymorphisme dans ces gènes chez des isolats de *P. falciparum* provenant de la province de Yunnan en Chine, nous avons génotypé le codon 76 de *pfcr* et les codons 86 et 1246 de *pfmdr1*. Nos résultats démontrent que bien que la QC ait été retirée du traitement de la malaria *falciparum* depuis plus de deux décennies, 90,3% des parasites portaient toujours la mutation K76T de *pfcr*. En revanche, les mutations au niveau des codons 86 et 1246 de *pfmdr1* étaient rares. L'analyse des séquences du gène *pfcr* chez 34 parasites isolés sur le terrain a indiqué un haplotype CVIET au niveau des positions 72–76 comme étant le type majeur, ce qui est consistant avec la théorie de l'origine sud-est asiatique de la résistance du parasite à la QC. En outre, deux nouveaux haplotypes de *pfcr* (75D/144Y/220A et 75E/144Y/220A) ont été identifiés. La réaction en chaîne de la polymérase en temps réel a été utilisée pour déterminer l'amplification du gène *pfmdr1*, associé à la résistance à la méfloquine. Nos résultats confirment cela en accord avec le fait que la méfloquine n'a pas été utilisée dans cet endroit. En effet, la plupart (>90%) des parasites avaient une seule copie de *pfmdr1*. Le génotypage de deux loci hypervariables a révélé des niveaux relativement faibles de la diversité génétique de la population de parasite. Toutefois, 28,4% de cas se sont avérés contenir des mélanges de clones, ce qui favorise la recombinaison génétique. En plus, malgré l'histoire unique des médicaments antimalariques à Yunnan, ses connexions géographiques avec trois pays malariques facilitent le transfert de gènes au sein des populations de parasites et l'évolution de nouveaux génotypes résistants aux médicaments. Par conséquent, la surveillance continue de la résistance aux médicaments dans ce secteur est nécessaire pour l'ajustement ponctuel des politiques locales sur les médicaments et pour un contrôle plus efficace de la malaria.

**mots clés** parasite de malaria, résistance à la chloroquine, *pfcr*, *pfmdr1*, diversité génétique

#### Análisis molecular de la resistencia a cloroquina en *Plasmodium falciparum* en la Provincia de Yunnan, China

La resistencia de *Plasmodium falciparum* a la cloroquina (CQ) está determinada por la mutación de K76T del gen del transportador de resistencia a cloroquina de *P. falciparum* (*pfcr*), y modificada por otras mutaciones de este gen, así como en el gen de multiresistencia 1 de *P. falciparum* (*pfmdr1*). Con el fin de determinar la extensión del polimorfismo de estos genes en aislados silvestres de *P. falciparum* de la Provincia de Yunnan en China, hemos genotipado el codon 76 de *pfcr*, los codones 86 y el 1246 de *pfmdr1*. Nuestros resultados muestran que, aunque hace más de dos décadas que se retiró la CQ como tratamiento para la malaria por *falciparum*, un 90.3% de los parásitos aún tiene la mutación K76T del *pfcr*. En cambio, las mutaciones en los codones 86 y 1246 del gen *pfmdr1* son raras. La secuenciación del gen *pfcr* en 34 parásitos de campo, reveló que el CVIET en las posiciones 72–76 es el tipo predominante, de forma consistente con la teoría del origen sureste-asiático de la resistencia a CQ en el parásito. Adicionalmente, se identificaron dos nuevos haplotipos de *pfcr* (75D/144Y/220A y 75E/144Y/220A). Se utilizó la PCR a tiempo real para determinar la amplificación del gen *pfmdr1*, la cual se asocia con la resistencia a mefloquina. Nuestros resultados indican que, en línea con el hecho de que la mefloquina no se ha utilizado en esta área, la mayoría de los parásitos (>90%) tenían una copia del *pfmdr1*. El genotipado de dos loci hipervariables mostró niveles relativamente bajos de diversidad genética en la población parasitaria. Por otro lado, se encontró que un 28.4% de los casos tenían clones mixtos, lo cual favorece la recombinación genética. Más aún, a pesar de la historia única de antimaláricos en Yunnan, las conexiones geográficas con tres países endémicos para malaria facilita el flujo genético entre las poblaciones de parásitos y la evolución de genotipos resistentes noveles. Por lo tanto, en esta área es necesario mantener una vigilancia continua de resistencia a antimaláricos, con el fin de ajustar a tiempo las políticas de medicamentos y ejercer un control de la malaria más efectivo.

**palabras clave** parásito de malaria, resistencia a cloroquina, *pfcr*, *pfmdr1*, diversidad genética